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Influences of Aged Bone Marrow Macrophages on Skeletal Health and Senescence

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Abstract

Purpose of Review The purpose of this review is to discuss the role of macrophages in the regulation of skeletal health with age, particularly in regard to both established and unexplored mechanisms in driving inflammation and senescence.

Recent Findings A multitude of research has uncovered mechanisms of intrinsic aging in macrophages, detrimental factors released by these immune cells, and crosstalk from senescent mesenchymal cell types, which altogether drive age-related bone loss. Furthermore, bone marrow macrophages were recently proposed to be responsible for the megakaryocytic shift during aging and overall maintenance of the hematopoietic niche. Studies on extra-skeletal macrophages have shed light on possible conserved mechanisms within bone and highlight the importance of these cells in systemic aging.

Summary Macrophages are a critically important cell type in maintaining skeletal homeostasis with age. New discoveries in this area are of utmost importance in fully understanding the pathogenesis of osteoporosis in aged individuals.

Keywords Aging · Immunosenescence · Myeloid · Macrophage · Senescence · Phagocytosis

Introduction

Osteoporosis is generally considered an age-associated disease and is prevalent in elderly individuals [1]. An estimated 25 million Americans over the age of 50 have osteoporosis with approximately 3 million fragility fractures occurring every year in the USA, leading to an annual economic cost of greater than $18 billion [2]. These osteoporotic fractures lead to a high clinical burden in the elderly, with patients aged 65 years or older exhibiting death rates of up to 36% within 12 months of a hip fracture [3]; this unusually high mortality rate is predicted to be driven by the existence of age-related comorbidities. As individuals in this age-group are expected to reach nearly 20% of the global population by 2050 [4], the need to understand the mechanisms of aging on osteoporosis development is more important than ever.

Macrophages are of utmost importance for the innate immune response [5]. The skeleton has a central role in macrophage physiology across the entire organism, as a large subset of these cells arise from circulating monocytes released from the bone marrow. These infiltrating macrophages surge in response to acute injury, including fracture, and have a multitude of functions that promote tissue repair. In addition, other bone-resident macrophages have demonstrated critical roles in maintaining skeletal homeostasis and have highly influential roles in bone resorption. These beneficial roles can turn detrimental upon the establishment of chronic inflammation in the setting of aging. Though the foundational concept of "inflammaging" has long been established, the mechanisms underlying the pathological roles of macrophages on skeletal health with age have only recently been revealed. In this review, we will discuss recent updates on roles of bone marrow macrophages in skeletal aging and senescence, while also touching on unexplored mechanisms that may be conserved from other extra-skeletal macrophage cell types.

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Age-Related Alterations in Bone Marrow Macrophage Subtypes

Extensive heterogeneity has been observed in bone marrow macrophages, revealing complex subtypes that differ in their origin, function, and plasticity. Within the bone microenvironment, the predominant tissue-resident macrophage-lineage populations include osteoclasts, osteal macrophages ("osteomacs"), and erythroblastic island macrophages [6]. Lineage tracing based on single-cell RNA sequencing data revealed that hematopoietic stem cells as well as yolk sac erythro-myeloid progenitors serve as bone-resident macrophage progenitors [7, 8]. Further differentiation into mature osteoclasts requires CSF1 signaling [9], similar to the differentiation of macrophages, along with NFκB signaling, such as RANKL [10]. In contrast with tissue-resident populations, iinfiltrating macrophages originate from common myeloid progenitors (CMPs) that are released into the blood as monocytes, travel to tissue sites, and then differentiate into macrophages [11]. They are generally divided into M1 (classically activated pro-inflammatory) and M2 (alternatively activated anti-inflammatory) subtypes. Activation via pro-inflammatory stimuli, like bacterial lipopolysaccharides (LPS), or high mobility group box 1 protein (HMGB1), leads to an pro-inflammatory response, causing the M1 macrophage to release a cocktail of pro-inflammatory cytokines and chemokines (e.g., IL-6, TNF-α, CXCL9, CXCL10) [12–14]. M2 polarization is induced through Th2 cytokines, like IL-4 and IL-13 [15], promoting the release of anti-inflammatory cytokines like IL-10 and TGF-β [15]. Furthermore, evidence suggests that pro-inflammatory macrophages are able to mute the anti-inflammatory phenotype [16]. It should be mentioned that this classification of macrophages has been criticized as simply painting the two extremes of the macrophage polarization spectrum and not being applicable in vivo [17]. However, due to its clarity and simplicity, the M1/M2 model has been established as a common working model for macrophages and will therefore also be used in this review.

With age, several differences in macrophages can be observed. During aging, an accumulation of inflammatory factors, as well as an altered inflammatory response can be observed, shown to arise from altered macrophage physiology [18, 19]. This chronic inflammatory state that occurs with aging is also known as "inflammaging." Although the puzzle behind this phenomenon is far from being solved, a number of mechanisms have been proposed. It has been reported that, upon stimulation with inflammatory factors such as LPS, macrophages express CD38, a NADase, and release it into the environment [20]. Nicotinamide adenine dinucleotide (NAD) is involved in redox reactions but also in other key processes, such as cell signaling and DNA repair [21]. Interestingly, a decrease in intracellular NAD with age has been observed, most likely due to inflammaging-induced upregulation of CD38 [22–24]. The decrease of NAD is associated with numerous age-related morbidities, as well as bone mass loss [25, 26]. CD38 has also been found to be specific for M1-like macrophages [27]. Accordingly, while the overall quantity of macrophages remained the same with age, aged mice exhibited increased expression of genes associated with pro-inflammatory M1-macrophages in fracture calluses, with unique overall transcriptomes relative to young mice [28]. Aged mice treated with a CSF1R inhibitor, thereby inhibiting macrophage recruitment, demonstrated improved fracture healing, whereas no effect was observed in young mice. Along these lines, it was found that M2 macrophages promote bone regeneration in the settings of heterotopic ossification and fracture [29]. This evidence outlines likely mechanisms by which age-related alterations in the makeup of macrophage subtypes disrupt skeletal metabolism.

Macrophage-Mediated Modulation of Bone Loss and Regeneration

Due to the abundance of myeloid lineage cells in the bone marrow, the crosstalk between macrophages and cells maintaining bone tissue integrity is of vast significance. This relationship has been suggested since the initial use of clodronate, a first-generation bisphosphonate, in osteoporotic patients [30], as this drug is also commonly used in mouse experiments to deplete macrophages [31]. In 2002, it was discovered that macrophages release osteogenic proteins, such as BMP-2, which promote osteoblast differentiation [32]. Since then, numerous functions of macrophages have been revealed in promoting the activities of bone formation [33–35], resorption [36], and fracture healing [37]. Conversely, others have found macrophages inhibit bone formation [28, 38], typically as a result of the release of pro-inflammatory interleukins, interferons, and other cytokines [39]. These contradicting results arise from macrophage diversity and/or plasticity, as different effects on skeletal cells may be cell type- or context-dependent. For instance, infiltrating macrophages with a pro-inflammatory "M1-like" phenotype have been demonstrated to drive inflammation with aging in both bone [28] and other tissues [40], while tissue-resident osteomacs have beneficial roles in regulating bone formation and resorption.

Osteomacs are unique to bone and have been reported to influence skeletal metabolism through a number of mechanisms [36]. There is a current debate about their specific cell surface markers; in mice, CD169 and tartrate-resistant

acid phosphatase (TRAP) expressing cells have been characterized as osteomacs, while other groups define them as F4/80 + Cd45 + cells [41, 42]. In previous research, CD68 has been used to show osteomac presence in human bone [43]. The close proximity of osteomacs towards other bone lining cells allows them to regulate bone remodeling. In vitro experiments reported that depletion of macrophage-lineage cells resulted in impaired matrix mineralization and reduced bone forming capacities [43]. Recent in vivo studies in mice depleting CD169 + macrophages confirm these results [41]. Furthermore, it was suggested that the reduced bone forming capacity is not caused by increased osteoclast activity but rather due to the paracrine influence of osteomacs on osteoblasts. The observed loss of osteoblasts after osteomac depletion leads to the conclusion that osteomacs promote osteoblast maintenance either through production of survival factors or other unknown mechanisms [41]. Their association with pathologic bone formation when present, and subsequent reduction of pathologic bone formation when depleted, points out their importance for bone formation [44, 45].

Interestingly, recent studies could provide in vivo evidence of apoptotic osteoblast clearance by macrophages [46]. While osteoblasts can develop into osteocytes or bone lining cells, a larger majority of osteoblasts undergo cell death [47]. Considering the suggested role of osteomacs in bone formation, as well as their role in apoptotic clearance, the question arises how mechanisms of clearance of apoptotic osteoblasts and new bone formation are linked [46]. Bone resorption and subsequent bone loss are also heavily influenced by macrophages. TNF-α and IL-1β, both secreted by macrophages, can drive osteoclast formation both directly as well as indirectly through inducing osteocytic production of RANKL [15, 48]. Osteomacs not only support bone resorption by osteoclasts, but also support clearance of the resorption residues [49]. As macrophages have been observed to secrete matrix-metalloproteinases (MMPs) in inflammatory states [50, 51], it is plausible that this also occurs in bone marrow macrophages and may contribute to age-related bone loss; however, this remains to be tested.

Crosstalk Between Macrophages and Aged Skeletal Cells—a Role for CSF1

Signals emanating from mesenchymal cells have important roles in macrophage recruitment and function. Macrophage colony-stimulating factor (CSF1/M-CSF), a growth factor that drives differentiation of myeloid lineage cells, has recently been of major focus in the bone field. CSF1 deletion was first implicated in bone when it was discovered that the cause of osteopetrosis in the *op/op* mouse model was due to a loss-of-function mutation in *Csf1* [52, 53], resulting in drastically reduced numbers of macrophages and osteoclasts [54]. Within the bone, osteocytes have been observed to release CSF1, and osteocyte-specific deletion of CSF1 led to increased bone mass and reduced osteoclasts, similar to global deletion [55]. Along with non-autonomous effects on myeloid cells, CSF1 was also observed to maintain osteocyte homeostasis both in vitro and in vivo through the inhibition of intracellular ROS production and apoptosis [55]. In addition to osteocytes, it was recently found — independently by two groups — that marrow-adipogenic lineage precursors (MALPs) are the primary source of CSF1 in the bone microenvironment [9, 56]. MALPs are defined by adipocytic gene expression, particularly *Adipoq*, yet exist as stromal and perivascular cells in the marrow cavity [57]. Both groups found that deletion of CSF1 in MALPs drastically reduced whole-marrow CSF1 expression and led to osteopetrosis and reduced bone marrow macrophage abundance upwards of 50%. Although the latter is commonly interpreted as the underlying reason for reduced osteoclast numbers, the resulting non-autonomous effects of reduced bone marrow macrophages on bone metabolism were not investigated. CSF1 in isolation is used to differentiate monocytes towards macrophages in vitro, requiring additional RANKL supplementation to generate osteoclasts [58]. A similar environment exists in the aging skeletal niche, as Ambrosi et al. found that skeletal stem cells (SSCs) upregulate CSF1 with age, but not RANKL [59]. Interestingly, as detailed further below, CSF1 has been implicated in cellular senescence in aged skeletal tissue, although its mechanism of action in age-related bone loss remains unclear. Thus, it will be important to understand the relevance of macrophage-specific CSF1 signaling in skeletal aging.

Macrophage Influence on HSC Niche

Hematopoetic stem cells (HSCs) reside within the bone marrow and are responsible for producing all blood and immune cells and maintaining them to adequate numbers. During their lifespan, they can experience various routes: They can become quiescent, apoptotic, self-replicate, differentiate into hematopoietic progenitors (HPCs), or even migrate [60]. They are tightly regulated by so-called "niches," specific microenviroments within the bone, where various cell types (e.g., HSCs, Osteoblasts) influence lineage development. Among other cell types, macrophages have an influential role in regulating these niches [61].

In vivo depletion of macrophages leads to a loss of osteoblasts and more importantly a loss of function of the niche, shown by HSC and HPC mobilization and migration into the periphery [61]. Furthermore, treatment with G-CSF, used to mobilize HSCs, caused osteomacs to disappear from endosteal surfaces, even before osteoblast depletion occurred [61]. This chain of events was remarkably similar to what happened upon depletion of macrophages, underlining their importance in maintaining the niche [61].

Aged myeloid-biased murine HSCs, marked by CD41 expression, also showed increased expression of von Willebrand factor (vWF) and other megakaryocytic genes, indicating a platelet shift (platelet bias) [62]. This skewing towards the megakaryocytic line could also be observed in humans with age [63]. Experiments have shown that aged niche macrophages were able to induce this HSC platelet bias, even in the presence of young macrophages, questioning the role of macrophages in aging HSC niche maintenance [64]. It is suspected that the shift is caused by several distinct mechanisms. Aged bone marrow macrophages shift towards inflammatory M1 phenotype, thus exhibiting increased production of IL-1β [64]. Additionally, through impaired phagocytosis of neutrophils, a main producer of IL-1β, an accumulation of IL-1β could be observed [64]. IL-1β is known to promote maturation of megakaryocytes and eventually of platelets [65]. It should be mentioned that in settings of global inflammation, a reduction of thrombocytes can be observed; thus, the question arises if the platelet bias in age might be beneficial in the context of inflammaging [66].

Osteomacs also maintain the hematopoietic niche in a manner unique to other macrophages [42]. Mohamad et al. found that, although osteomacs and bone marrow macrophages co-express similar markers (e.g., CD45, F4/80, CD11b), a unique osteomac population defined as CD166 + CSF1R + supported HSC function. This is in contrast with previous studies, as it was shown that CD169 and CD68, traditional markers for osteomacs, were also expressed by bone marrow macrophages. Interestingly, substitution of BMDMs for osteomacs in multicellular co-cultures failed to fully support the HSC niche, suggesting that osteomacs are required for the hematopoietic-enhancing activity of osteoblasts and show a unique response upon megakaryocyte stimulation [42].

As CD166 was identified as critical for maintaining the niche and was also expressed on HSCs, it is likely that these cells have a key regulatory function within the HSC niche [67]. Furthermore, osteomacs have been shown to be required for the hematopoietic-enhancing activity of osteoblasts [42]. Overall, osteal and bone marrow-derived macrophages have been shown to be of utmost importance as regulators within the HSC niche. Nonetheless, the HSC niche and the role of macrophages therein remain uncertain and further research is required to definitively describe their role.

Macrophages and Cellular Senescence

Cellular senescence is a state of growth-arrest concomitant with release of inflammatory cytokines that drive tissue dysfunction and disease [68]. Senescent cells have been shown to accumulate with age in both mouse and human skeletal tissue [69], and clearance of senescent cells in aged mice can delay age-related bone loss [70]. Several characteristics of senescence have been tightly implicated in the physiology of macrophages, which raises important questions regarding their involvement in senescence-driven states of bone loss.

Many macrophage signaling factors have been established in the senescence-associated secretory phenotype (SASP), which suggests that macrophage recruitment and differentiation are promoted by senescent cells. Although originally identified in senescent fibroblasts [71] and tumor cells [72], these SASP factors were only recently linked to senescence in the skeleton [69, 73]. Recently, Saul et al. established a SASP geneset using RNA-seq data from bone biopsies of two aging human cohorts, which was then validated in mouse single-cell RNA-seq samples. Over a fourth of this SASP panel consists of cytokines and chemokine families (e.g., CC & CXC family chemokines, interleukins) with macrophage modulatory functions. Furthermore, SASP-associated cells were found to be highly enriched for MHC-I signaling. In addition to paracrine signaling, it was recently found that senescent cells directly bind to macrophages through their expression of CD47, which inhibits efferocytosis, thereby disrupting tissue homeostasis [74]. As the presence of senescent cells contributes to age-related bone loss, it is plausible that this deleterious phenotype is at least partially driven by their modulation of macrophage function. This has been well-documented in other disease settings, such as cancer [72, 75–77]. However, the extent to which senescence-driven recruitment, differentiation, or polarization of macrophages is able to drive age-related bone loss remains to be tested.

Along with non-autonomous signaling to macrophages, it has been proposed that macrophages themselves may become senescent with age, thereby driving tissue disease. Senescent macrophages have been studied in the context of cancer, whereby they drive tumor progression through a secretory phenotype, and targeting senescent cells can alleviate this phenotype [78, 79]. This phenotype has only recently been linked to skeletal physiology. A recent study by Li et al. concluded that senescent macrophages and neutrophils secrete grancalcin (GCA), which drives age-related bone loss [80]. Grancalcin was found to be upregulated with age and inhibited signaling of the plexin-b2 receptor on mesenchymal cells, thereby downregulating osteogenesis and impairing bone formation. This study established a signaling axis between aged macrophages and mesenchymal cells in regulating bone loss, despite one issue. Although it was concluded that grancalcin was produced by bone marrow macrophages that were senescent, their single-cell

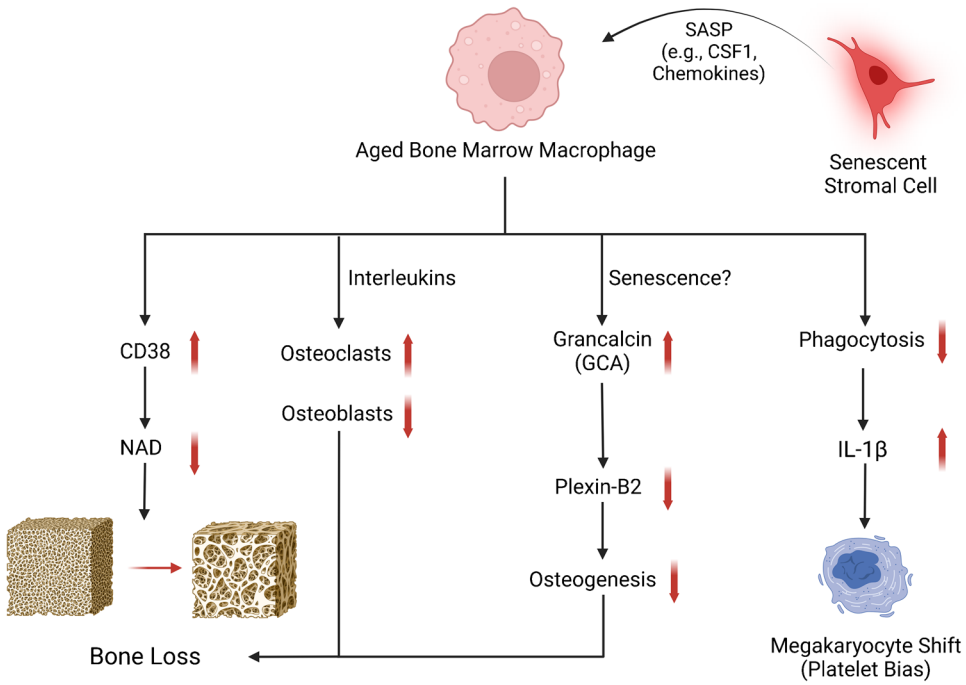

Fig. 1 Aged bone marrow macrophages disrupt skeletal metabolism through newly discovered mechanisms. Macrophages have been reported to disrupt the balance of bone formation and resorption with age through intrinsic alterations in macrophage polarization (e.g., CD38 upregulation), promoting osteoclastogenesis, and release of grancalcin upon developing a senescent-like phenotype. Macrophages also disrupt the HSC niche through release of IL-1β and can be influenced by the inflammatory niche generated by aged and senescent skeletal cells. SASP, senescence-associated secretory phenotype. Created with BioRender.com

analyses revealed that these cells also demonstrated high *Mki67* expression, suggesting they are proliferative. This suggests that, although these macrophages were highly inflammatory, they may not have been truly senescent. This unfortunately is a well-documented occurrence in macrophages, as they can express senescent markers (e.g., express p16 and stain positive for senescence-associated β-galactosidase), yet this is simply part of a senescence-independent physiological response [81]. Moreover, the inflammatory nature of certain macrophage types overlaps greatly with SASP proteins. The difficulties of differentiating senescence from non-senescence in aged macrophages have been discussed at length [82], and will be a significant hurdle in the study of macrophages in senescence-driven states of bone loss. Regardless of whether or not macrophages truly become senescent, it is clear that their secretory profile can have damaging effects on nearby skeletal cells.

Conclusions

Macrophages within the bone microenvironment have diverse and important functions in regulating bone mass, in addition to their established roles in innate immunity. Macrophage functions have traditionally been studied within the realm of osteoclast differentiation, yet it has been revealed that these cells impress non-autonomous effects upon numerous mesenchymal cell types within the bone microenvironment. Additionally, aged skeletal cells release factors involved in macrophage recruitment, polarization, and differentiation, which implicate these immune cells in states of aging and senescence.

Although much has been revealed (Fig. 1), several important questions remain. Firstly, although the heterogeneity of infiltrating and tissue-resident macrophage subpopulations in the bone marrow have been classically described, their complexity remains to be elucidated at the single-cell level, as has been done for skeletal muscle macrophages [83, 84]. A major reason this has yet to be accomplished is due to the fragmentation of bone-resident macrophages upon traditional isolation for cytometry [85], which may be circumvented by negative selection techniques or spatially resolved approaches. Secondly, the osteoclast-independent effects of macrophages in driving age-related bone loss require further elucidation, particularly in the setting of CSF1 released by aged SSCs [59]. As CSF1 and other macrophage-related cytokines are additionally released by senescent cells as part of the SASP, establishing a link between the well-documented functions of these cytokines and unexplored age-related cellular mechanisms would be of substantial importance. Thirdly, as described in the previous section, it remains to be observed the extent to which bone marrow macrophages become truly senescent. As many characteristics are shared between senescent cells and terminally differentiated macrophages [82], careful characterization of these immune cells will be required to accurately implicate these cells in senescence-driven disease states.

Although macrophages have been studied for decades, new roles for these cell types are being discovered every

year, implicating them in various disease states. As such, the role of these cells in age-related bone loss will be of great interest in fully understanding the pathophysiology of osteoporosis in elderly patients.

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Declarations

Conflict of Interest The authors declare no competing interests.

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